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REGULATION OF CELLULAR OSMOLARITY AND VOLUME IN *TETRAHYMENA*

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INTRODUCTION

Freshwater Protozoa are hyperosmotic to their environment. Direct determinations of intracellular osmolarity on *Amoeba proteus* and *Pelomyxa carolinensis* have given values between 101 and 117 m-osmole/kg cells, when the external osmolarity was 2–8 m-osmole/l (Løvtrup & Pigon, 1951; Riddick, 1968; Schmidt-Nielsen & Schrauger, 1963). No measurements were made of intracellular osmolarity in response to changes in the medium. There have been no reliable direct measurements of osmolarity on ciliates, but there is evidence as to what solutes are responsible for the intracellular osmolarity. Most of the evidence available is for the concentrations of sodium, potassium, chloride and phosphates (orthophosphates and phosphate esters) in *Tetrahymena pyriformis*, and these could account for *c.* 75 m-osmole/kg cells (Dunham, 1970; Cline & Conner, 1966). Attempts have been made to quantify the free amino acids of *Tetrahymena*, and the results suggest that they may be important osmotic constituents as well (Scherbaum *et al.* 1959; Wu & Hogg, 1956). In the marine ciliate *Miamiensis avidus* in sea water the intracellular free amino acids constituted 300 mM/kg cells, a large fraction of the total intracellular osmolarity. The amount of free amino acids/cell was found to increase or decrease in response to corresponding changes in the external osmolarity (Kaneshiro, Holz & Dunham, 1969).

The purpose of the present study was to determine the intracellular osmolarity of *Tetrahymena pyriformis* and the relationship between the internal and external osmolarities. The contribution of the free amino acids to the intracellular osmolarity was also determined. In the most dilute media the intracellular osmolarity of *Tetrahymena* is 111 m-osmole/kg cells; determined from the freezing-point depression of cell extracts. About 90% of the intracellular osmolarity could be accounted for by sodium, potassium, chloride and the free amino acids. When the external osmolarity was increased or decreased there were corresponding changes in the amounts of the intracellular solutes, principally amino acids. Regulation of cell volume and the activity of the contractile vacuole could be correlated with the change in the amounts of intracellular free amino acids under conditions of osmotic stress.

MATERIALS AND METHODS

Tetrahymena pyriformis strain W were grown axenically in a culture medium containing 1·75% proteose peptone (Difco) and 0·25% yeast extract (Difco). Cultures were grown in 2800 ml Fernbach flasks containing 500 ml of the culture medium at 23 °C.

The cultures were harvested after 4 days of growth by centrifugation at 150 *g* for 30 s. The yield from one of these cultures ranged between 2 and 3 g of cells, wet weight. Mean cell volumes were calculated from the wet weight of packed pellets centrifuged from 50 ml of cell suspension, and the mean number of cells/ml of the cell suspension, determined in a Sedgewick–Rafter counting chamber. The values were corrected for extracellular space, determined by a method given below. The mean cell volume at the time of harvesting was 28·3 pl/cell; after 12 h in an inorganic medium (composition given below) the cell volume had decreased to 10·2 pl/cell.

After harvesting, the cells were subjected to a change in the external osmolarity. At appropriate times samples were taken for determination of freezing-point depression, free amino acids, inorganic ion concentrations, and cell volume. The external osmolarity was varied by one of several methods: by dilution of the growth medium with distilled water, or by washing the cells by gentle centrifugation in solutions of various sucrose concentrations buffered at pH 6·8, with 1 mM/l sodium phosphate buffer. Samples were taken after the cells had been exposed to these media for 4–5 h. In other experiments the cells were washed twice in an inorganic medium containing 30 mM/l NaCl, 5 mM/l KCl, 0·5 mM/l $CaCl_2$, 0·5 mM/l $MgSO_4$, and 1 mM/l Na phosphate buffer at pH 6·8. The cells were allowed to stand 12 h before a final wash in the inorganic medium. Then sucrose was added to the cell suspension to raise the external osmolarity. In all experiments the number of cells/unit volume of medium was maintained near that in the growth medium at the time of harvesting.

For determination of freezing-point depression, duplicate/samples of packed cells were obtained by centrifuging 50 ml of the cell suspension at 150 *g* for 30 s, and aspirating off all but 8 ml of the supernatant. The cells were re-suspended, placed in a 10 ml Kolmer tube, and centrifuged at 1100 *g* for 6 min. The supernatant was saved, the tubes were wiped dry, weighed, and immediately placed in a beaker of boiling water for 5 min. About 2 min elapsed from the end of centrifugation until the placing of the tubes in boiling water. The above method of preparing samples for freezing-point determination is a modification of a method used in mammalian tissues (Appleboom, 1958).

The pellets, weighing 100–200 mg, were kept frozen until immediately before the determination of freezing points, usually 1 day later. At this time the pellets were re-suspended in 100–200 μl of distilled water and placed again in a beaker of boiling water for 5 min. The tubes were centrifuged again for 3 min at 1100 *g*, and weighed to determine the extent of evaporation, which was typically 30 mg of water. A glass pipette was used to place *c.* 3 nl of the clear supernatant in the sample plate of a Clifton Nanoliter Osmometer (Clifton Technical Physics, Wanamassa, N.J.). The freezing point was determined for the samples, for distilled water, and for a sodium chloride standard whose freezing-point depression was 1 °C (17·1 g NaCl/kg water). It was assumed that 1 mole/kg water of an ideal solute would depress the freezing point of distilled water by 1·86 °C under STP conditions. The data are reported in mM of solute/kg cells, taking into account the extracellular space and dilution of the extracts. When the cell volume changed during the course of an experiment the data are also given as mM solute in the number of cells which constituted 1 kg at the beginning of the experiment, $8{\cdot}52 \times 10^{10}$ cells. The data in this form corresponds to the amount of solute/cell and not the concentration.

The validity of the above technique for determining the intracellular osmolarity (the total concentration of solutes in the cells as determined by freezing-point depression) was tested in two ways. First, the time between the end of centrifugation and first immersion in boiling water was varied to determine whether autolysis of cell constituents in the packed cells increased the osmolarity of the pellet. It was found that the osmolarity of the pellet did increase slightly as the time of boiling was delayed but by less than 5% in 2 min. This error was considered minor and the data have not been corrected for it. Secondly, we determined whether dilution of the cytoplasmic solutes, when water was added to the boiled pellets, altered their effective concentration by increasing the activity coefficients. Accordingly, 0–500 μl of distilled water were added to a series of extracts of 200 mg pellets and their freezing points were determined. The calculated intracellular osmolarities were the same for all of the dilutions of the pellets.

Concentrations of intracellular ions were determined on acid extracts of packed pellets of cells (Dunham, 1964). Sodium and potassium measurements were obtained with an IL model 143 flame photometer (Instrumental Laboratories Inc., Lexington, Mass.). An Aminoc–Cotlove titrator was used to measure the concentrations of chloride. Extracellular space of packed pellets was determined after adding 0·6 μCi of [^{14}] C inulin (New England Nuclear) to 50 ml of cell suspension immediately prior to centrifugation. The radioactivity of samples of the pellet extracts and the medium were determined in Bray's solution (Bray, 1960) in a Packard Liquid Scintillation Counter. The mean extracellular space of four packed pellets of cells exposed to inorganic medium for 12 h was 15·2%. The mean extracellular space of cells in inorganic medium plus 100 mM sucrose for 1 h was 16·8% (n = 4). All data presented in this paper are corrected for extracellular space.

Free amino acids were extracted from 1 ml of packed pellets with 70% ethanol. A norleucine standard was introduced for the eventual determination of the fraction of the extracted material recovered. Samples were prepared for analysis by the technique of Kaneshiro *et al.* (1969). After the samples were brought to near dryness, they were re-suspended in a pH 2·2 sodium citrate buffer for analysis on a Beckman model 116 amino acid analyser. A 69 cm column of Aminex A-4 resin (Biorad) was used with a buffer system selected for single-column analysis. Colour factors and the concentrations of 21 amino acids (including norleucine) were determined using an amino acid standard solution containing equimolar concentrations of all of the amino acids (General Biochemicals). Eighteen amino acids were identified in the cell extracts by their elution times. Concentrations were calculated from colour factors and from the areas of the elution peaks. At least six unknown peaks were also present, but were not included in the concentration of the total free amino acids. The data are presented as mM of free amino acids/$8{\cdot}52 \times 10^{10}$ cells, the number of cells which constituted 1 kg in the inorganic medium.

The rate of fluid elimination by the contractile vacuole was determined on individual cells from the pulsation rate of the vacuole and its diameter just prior to systole. Individual cells were observed using the oil-immersion objective of a light microscope. Each determination represents measurements on five consecutive vacuolar cycles. The results are given in μm^3/min × cell.

To determine if any observed changes in cell volume during osmotic stress could be

attributed to sucrose entry, the extent of sucrose penetration into cells was measured in inorganic medium with 100 mM sucrose and [^{14}]C sucrose (New England Nuclear Corp.). Acid extracts of packed pellets were obtained at appropriate times and the amounts of [^{14}C]sucrose present in the medium and the pellet extracts were determined in Bray's solution (Bray, 1960) with a Packard Liquid Scintillation Counter. The rate of sucrose entry was linear with time for 3 h. After 1 h the sucrose concentration in the cells was only 7·1 % of that of the medium.

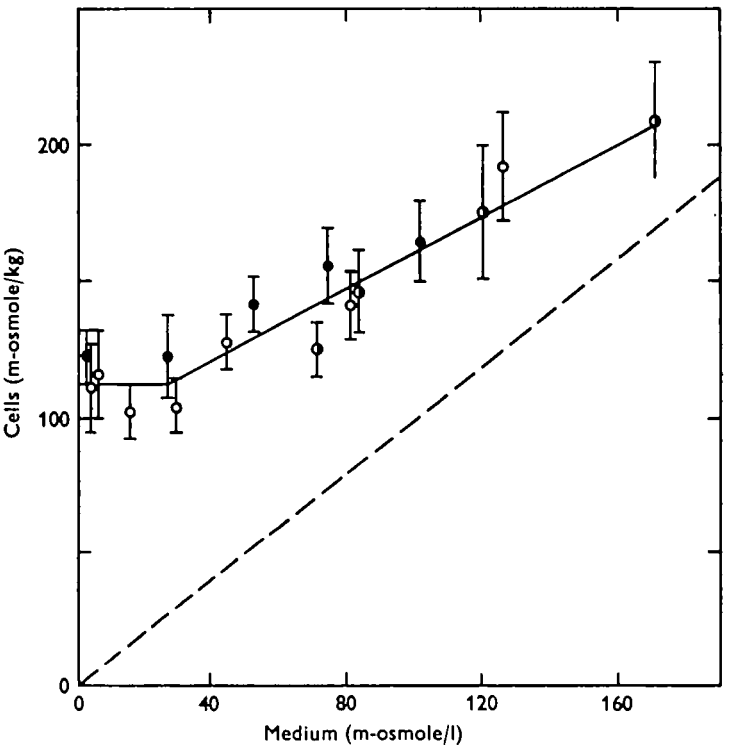

Fig. 1. The intracellular osmolarity of *Tetrahymena* in media of various osmolarities. Both intracellular and external osmolarities were calculated from freezing-point depressions. The results are expressed as m-osmole/kg cells for cells which had been exposed to: (○) dilutions of culture medium for 4–5 h; (●) low ionic strength solutions of various sucrose concentrations for 4–5 h; (◑) inorganic medium for 12 h prior to a 2 h exposure to inorganic medium (see text for composition) plus various concentrations of sucrose. Each symbol indicates the mean of ten determinations, (five experiments) ± 2 S.E.M. The dashed line connects points of equal cellular and external osmolarities.

RESULTS

The intracellular osmolarity of *Tetrahymena* was determined after exposure of the cells to media of various osmolarities. Data obtained for cells subjected to osmotic stress by three different methods are presented in Fig. 1. First, the external osmolarity was varied by dilution of the growth medium with distilled water, 4–5 h prior to the time the samples were taken (open circles). Secondly, samples were taken of cells in buffered sucrose solutions of low ionic strength for 4–5 h (closed circles). Finally, the half-closed circles represent the intracellular osmolarity of cells which were exposed

12 h to inorganic medium before the addition of sucrose to the medium. At each external osmolarity the results are substantially the same for all three conditions. The osmolarity of the cells remains constant over a range of low external osmolarities. At high external osmolarities the intracellular osmolarity is elevated linearly with increases in the external osmolarity. Other experiments show that there is no difference in results when cells are exposed to inorganic medium 3 h instead of 12 h prior to the addition of sucrose.

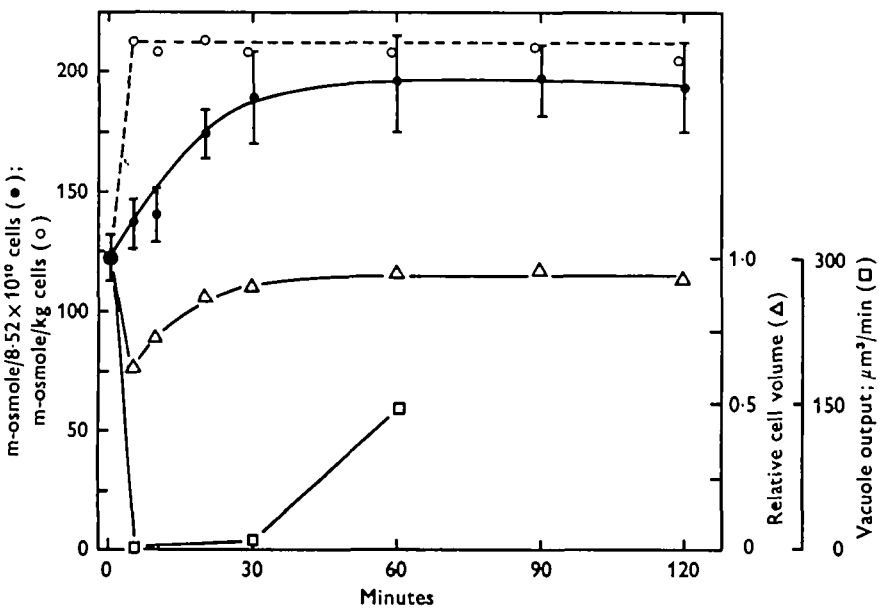

Fig. 2. The regulation of intracellular osmolarity and cell volume by *Tetrahymena* exposed to an osmotic stress, exposure to 100 mM sucrose. Cell suspensions were in inorganic medium for 12 h prior to the addition of sucrose. (○) the intracellular osmolarity (from freezing points); (△) the relative cell volume; (●) the amount of solute in mM/8·52 × 10^{10} cells (calculated from osmolarity and relative cell volume); the brackets show ± 2 S.E.M.; (□) the rate of fluid elimination by the contractile vacuole in μm^3/min × cell. Each symbol indicates the mean of ten determinations from five experiments. The symbols for all measurements coincide at time zero, as indicated by the large circle.

Fig. 2 shows the kinetics of the change in the intracellular osmolarity and relative volume of cells which had been in inorganic medium for 12 h and then were subjected to the addition of 100 mM sucrose to the inorganic medium. Also shown are the amounts of solute/unit number of cells, calculated from intracellular osmolarities and the relative cell volumes. The initial rapid increase in osmolarity is due to the decrease in cell volume. The amount of solutes/unit number of cells increases slowly and is accompanied by an increase in the cell volume. The cell volume after 5 min probably does not represent the minimum volume but it was not feasible to obtain earlier measurements.

Fig. 2 also shows the time course of the changes in rate of fluid elimination by the contractile vacuole (in μm^3/min × cell) after exposure to 100 mM sucrose. This was determined in separate experiments on cells treated in exactly the same manner as

above. During the first 20 min of exposure to sucrose no functioning contractile vacuoles were seen in any of the 50 cells observed. Vacuolar activity is detectable at 30 min after exposure to 100 mM sucrose and by 60 min has increased to nearly one-half its initial value.

Table 1. *Amounts of solutes, both individually measured and calculated from freezing-point data, in* Tetrahymena *in inorganic medium before and after exposure to* 100 mM *sucrose*

(The results are expressed in mM/8·52 × 10^{10} cells, the number of cells which constituted 1 kg before the addition of sucrose. The amounts of free amino acids represent the sum of 18 identified. *n* is the number of determinations.)

		mM/8·52 × 10^{10} cells		
Solutes	*n*	Inorganic medium	Inorganic medium + 100 mM sucrose (20 minutes)	Increase
Sodium	9	6·5	15·8	9·3
Potassium	9	28·8	34·4	5·6
Chloride	5	9·2	9·0	−0·2
Free amino acids	2	56·5	114·9	58·4
Total solutes				
Individually measured	—	101	174	73
From freezing-point data	12	123	174	51

Table 1 shows the amount/unit number of cells of the major intracellular solutes: sodium, potassium, chloride and the total free amino acids (the sum of 18 individually determined amino acids). Just as in fig. 2 the cells were exposed to inorganic medium for 12 h, and then to inorganic medium plus 100 mM sucrose for 20 min. The sum of the concentrations of intracellular solutes can account for 90% of the osmolarity predicted by freezing-point depression. Most of the increase in the amount of solutes/unit number of cells caused by increasing external osmolarity can be attributed to an increase in the concentrations of the free amino acids. The concentrations of glutamic acid, glycine and alanine represent 34·5% of the total free amino acids of cells in inorganic medium. The increase in the concentrations of these three amino acids accounts for 77% of the total change of concentration of amino acids.

DISCUSSION

In the most dilute media, the intracellular osmolarity of *Tetrahymena pyriformis* was 111 m-osmole/kg cells, a value very similar to those obtained by direct determinations of the osmolarity of amoebae in dilute media (Løvtrup & Pigon, 1951; Riddick, 1968; Schmidt-Nielsen & Schrauger, 1963). A number of indirect determinations have estimated the osmolarity of various protozoa to be between 50 and 160 m-osmole/l (equivalent to 25–80 mM-NaCl) (Kitching, 1967).

Tetrahymena maintains a relatively constant osmolarity over a range of low external concentrations but in higher external osmolarities the intracellular osmolarity increases linearly as the external osmolarity is increased. However, the difference between the

internal and external osmolarities decreases as the external osmolarity is raised. This pattern of osmoregulation is similar to that of numerous freshwater metazoans (Potts & Parry, 1964).

The effects of varying the external osmolarity on the intracellular osmolarity were essentially the same for dilutions of the growth medium and for media of both high and low ionic strength in which the osmolarity was varied with sucrose. Therefore, the changes in intracellular osmolarity in response to external changes are probably due to the total concentration of solute in the medium and not to changes of particular constituents. This same conclusion was reached for the effect of external changes on the concentrations of total free amino acids in *Miamiensis avidus*, a marine ciliate (Kaneshiro *et al.* 1969).

The return of the cell volume to its original level after the initial shrinkage in 100 mM sucrose cannot be attributed to sucrose entry since the recovery of volume is nearly complete in 30 min and sucrose enters the cells to the extent of only 3·5% of the external concentration in this time. Therefore, the recovery of cell volume is the result of active regulatory processes (Dunham, 1970). The results of the present study indicate two aspects to the mechanism of the regulation of cell volume. First, there is a cessation of the activity of the contractile vacuole and a resultant decrease in the rate of the unidirectional efflux of water from the cell. The activity of the contractile vacuole resumes only when the volume of the cell has returned to near its initial value. Secondly, there is an increase in the amount of osmotically active solutes in the cytoplasm, which results in an increase of the passive influx of water. Thus, by actively decreasing its water loss and increasing its water influx, *Tetrahymena* is able to return to the initial cell volume after a passive change in volume. The ability to attain the initial volume implies the existence of a feedback control, system relating vacuolar function, solute concentration, and volume. Nothing is known about the particular nature of this control system.

It has been suggested that the presumed hyperosmolarity and resultant contractile vacuole activity of *Miamiensis avidus* is necessary for the excretion of certain cell solutes (Kaneshiro *et al.* 1969). Riddick's work in *Pelomyxa carolinensis* has shown that the fluid of the contractile vacuole is hyposmotic to the cytoplasm and is relatively high in sodium and low in potassium (1968). It has been shown that the contractile vacuole of *Tetrahymena* excretes sodium from the cell (Dunham, 1970). These data suggest that *Tetrahymena* maintains itself hyperosmotic to its environment because a functional contractile vacuole is necessary for the secretion of certain intracellular solutes.

The total amount of intracellular solutes in *Tetrahymena* in the inorganic medium as determined by freezing-point depression was 123 mM/$8{\cdot}52 \times 10^{10}$ cells. The sum of inorganic ions and free amino acids analysed was 101 mM/$8{\cdot}52 \times 10^{10}$ cells, 82% of the total solutes. These two values may not be significantly different. On the other hand, there may be solutes which were not analysed (e.g. calcium, orthophosphate, and the six unidentified amino acid peaks). The fact that the sum of the concentrations of the inorganic ions and the free amino acids of cells which have been in the inorganic medium plus 100 mM sucrose for 20 min is identical to the value for total intracellular solutes as determined by freezing-point depression suggests that these values are not significantly different.

During the return of cell volume to near the initial volume after osmotic stress the

increase in the amount of total free amino acids, accounts for nearly 85% of the total change in the amount of intracellular solutes/unit number of cells 20 min after the addition of sucrose. It was found that the sum of the concentrations of glycine, alanine and glutamic acid constituted 34·5% of the total concentration of free amino acids of cells in inorganic medium. Earlier work on quantification of ethanol-soluble amino acids of *Tetrahymena* report that these three amino acids constituted 33% of the total (Scherbaum, 1959). In our work these amino acids accounted for 77% of the total change in concentration of free amino acids. Preliminary data from experiments in which the intracellular amino acids were labelled with [^{14}C]amino acids suggest that the increase in the total free amino acid concentration is the result of degradation of cellular polypeptides. A detailed report on the concentration of the individual amino acids will be published.

Changes in the amounts of potassium and chloride did not account for any significant portion of the increase in osmotically active solutes. Sodium increased 9·3 mM/$8{\cdot}52 \times 10^{10}$ cells, roughly 15% of the total change in the amount of solutes present. This increase in sodium is presumably due to the fact that sodium is normally secreted by the contractile vacuole, which at this time is not functional (Dunham, 1970). Although calcium (7 mM/kg) and orthophosphate (5 mM/kg) are found in relatively high concentrations in *Tetrahymena* (Dunham, 1970; Cline & Conner, 1966), they are probably not osmotically active, since a large portion of Ca and orthophosphate seem to exist in an insoluble form in *Tetrahymena* (Rosenberg & Munk, 1969).

One cannot be certain that the total concentration of all the solutes, measured from concentrations of individual solutes or from freezing points, corresponds to the osmotic pressure in the intact cells. Because of changes in activity coefficients the solutes present may not exert their calculated osmotic effects within the cell. The same can be said for the reflexion coefficients of these solutes. On the other hand, the osmotically active solutes may not be evenly distributed throughout the cell water. This would tend to cancel the effects of activity coefficients and reflexion coefficients. We have not attempted to account for these potential sources of error in our data.

SUMMARY

1. *Tetrahymena pyriformis* are hyperosmotic to external media of osmolarities from 2 to 171 m-osmole/l. The intracellular osmolarity, determined by freezing-point depression, is 111 m-osmole/kg cells in dilute media, and increases linearly with increasing external osmolarities.

2. Over 80% of the intracellular osmolarity can be attributed to the concentration of sodium, potassium, chloride and the free amino acids.

3. In response to an increase in the external osmolarity, *Tetrahymena* regulates its intracellular osmolarity by increasing the concentrations of free amino acids.

4. The regulation of cellular volume under conditions of osmotic stress is achieved by an increase in the amount of osmotically active solutes and the regulation of the rate of elimination of fluid by the contractile vacuole.

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